

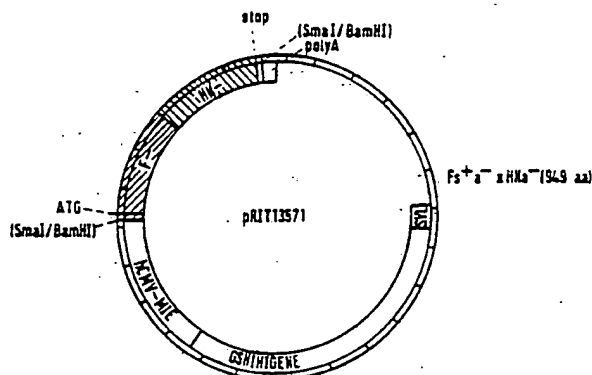
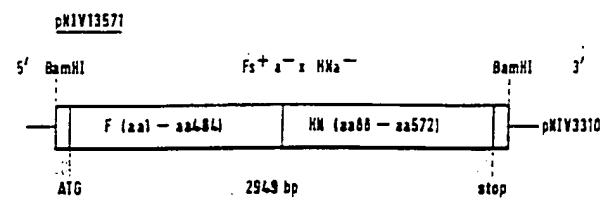
PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<p>(51) International Patent Classification ⁵ : C12N 15/45, 15/62, 15/53 A61K 39/155</p>	<p>A2</p>	<p>(11) International Publication Number: WO 93/06218 (43) International Publication Date: 1 April 1993 (01.04.93)</p>
<p>(21) International Application Number: PCT/EP92/02174 (22) International Filing Date: 18 September 1992 (18.09.92) (30) Priority data: 9120221.8 23 September 1991 (23.09.91) GB (71) Applicant (for all designated States except US): SMITH-KLINE BEECHAM BIOLOGICALS (S.A.) [BE/BE]; 89, rue de l'Institut, B-1330 Rixensart (BE). (72) Inventors; and (75) Inventors/Applicants (for US only): GHEYSEN, Dirk [BE/BE]; SmithKline Beecham Biologicals (s.a.), 89, rue de l'Institut, B-1330 Rixensart (BE). BOLLEN, Alex [BE/BE]; Gaasbeekstraat 65, B-1701 Ifterbeek (BE). BLAISE, Lydia [BE/BE]; 58, rue Saule Gaillard, B-4140 Jehay-Bodegne (BE).</p>		<p>(74) Agent: DALTON, Marcus, Jonathan, William; Smith-Kline Beecham, Corporate Patents, Great Burgh, Yew Tree Bottom Road, Epsom, Surrey KT18 5XQ (GB). (81) Designated States: AU, CA, JP, KR, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, SE). Published <i>Without international search report and to be republished upon receipt of that report.</i></p>

(54) Title: CHIMERAS OF PARAINFLUENZA VIRUS TYPE 3 PROTEINS F AND HN AS VACCINES



The invention relates to recombinant PIV3 fusion proteins or immunogenic derivatives thereof comprising a portion of the F protein of PIV3 fused to a portion of the HN protein of PIV3, methods of constructing and expressing these, intermediates for use therein and recombinant proteins obtained from the intermediates. Recombinant proteins of the invention have potential utility in the prevention of PIV3 infection.

ATTORNEY DOCKET NUMBER: 7682-055-999
SERIAL NUMBER: 09724.379
REFERENCE: C1

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FI	Finland	MN	Mongolia
AU	Australia	FR	France	MR	Mauritania
BB	Barbados	GA	Gabon	MW	Malawi
BE	Belgium	GB	United Kingdom	NL	Netherlands
BF	Burkina Faso	GN	Guinea	NO	Norway
BG	Bulgaria	GR	Greece	NZ	New Zealand
BJ	Benin	HU	Hungary	PL	Poland
BR	Brazil	IE	Ireland	PT	Portugal
CA	Canada	IT	Italy	RO	Romania
CF	Central African Republic	JP	Japan	RU	Russian Federation
CG	Congo	KP	Democratic People's Republic of Korea	SD	Sudan
CH	Switzerland	KR	Republic of Korea	SE	Sweden
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovak Republic
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CS	Czechoslovakia	LU	Luxembourg	SU	Soviet Union
CZ	Czech Republic	MC	Monaco	TD	Chad
DE	Germany	MG	Madagascar	TG	Togo
DK	Denmark	ML	Mali	UA	Ukraine
ES	Spain			US	United States of America

- 1 -

CHIMERAS OF PARAINFLUENZA VIRUS TYPE 3 PROTEINS F AND HN AS VACCINES

The present invention relates to recombinant PIV 3 proteins, more particularly to the F and HN proteins, fusion proteins derived therefrom, and their expression in eukaryotic cells. The invention further relates to methods of constructing and expressing the said fusion proteins, intermediates for use therein and recombinant proteins which may be obtained from the intermediates. Recombinant proteins of the invention have potential utility in the development of vaccines for the prevention of PIV 3 infection.

Parainfluenza viruses are important pathogens of the respiratory tract in infants and young children. Some infections are not restricted to the upper respiratory tract but also invade the lower part of the respiratory system, particularly in children under 5 years of age. For a review see Parainfluenza Viruses (chapter 35); Channock, R.M. and McIntosh, K. (1990) Virology, Second Edition, edited by B.N. Fields and D.N. Knipe. Raven Press, Inc. N.Y.; pp 963-988.

Together all parainfluenza virus infections are second only to those caused by the respiratory syncytial virus. PIV3 infections, in particular, are not only associated with the croup syndrome (as in the case of PIV1 and PIV2) but more often cause bronchitis, bronchiolitis and pneumonia. PIV3 is responsible for most of the fatal infections observed with paramyxoviruses. In particular, severely immuno-compromised children are at risk of developing fatal giant cell pneumonia when infected with PIV3. (Jarvis, W.R.; Middleton, P.J. and Gelfand, E.W. (1979). J. Pediatr. 94, 423-425)

Almost 67% of infants are infected with PIV3 in the first year of life; by age 2, 92% of children have been infected at least once by PIV3 and 37% have been infected more than once. The first infection is most likely to cause lower respiratory tract diseases. (Glezen, W.P.; Frank, A.L.; Taber, L.H. and Kasel, J.A. (1984). J. Infec. Dis. 150, 851-857)

No efficient vaccine against PIV3 is available; earlier attempts with inactivated PIV3 virus failed to induce resistance to the disease. New strategies currently being pursued include the use of cold-adapted

- 2 -

mutants of PIV3, the use of a bovine PIV3 and the development of subunit vaccines. The present invention relates to this last approach.

5 The fusion protein F of PIV3 contains 539 amino acid residues; amino acids 1 to 18 correspond to the signal peptide and residues 494 to 516 to the membrane anchor domain. The molecule presents potential sites for glycosylation. The F protein is synthesized as a 70 kDa precursor (F₀) which undergoes proteolytic maturation to yield the F₁ (56kDa) and F₂ (14 kDa) subunits linked via disulfide bridges. The protein F, when
10 injected into animals, leads to the production of neutralizing antibodies. The F protein is involved in cell fusion during viral infection and carries an hemolysin activity. Used alone for immunization, the F protein generates an immune response which is insufficient to confer protection against a challenge with the virus. Complete protection is only acquired
15 by concomitant immunization with the protein HN, another glycoprotein of PIV3.

The protein HN carries hemagglutinin and neuraminidase activities. It is composed of 572 amino acids; its membrane anchor domain occurs in the
20 N-terminal end of the molecule, between amino acid residues 32 and 53. Four potential sites for glycosylation have been identified. Injection of protein HN into animals generates an immune response and neutralizing antibodies. These antibodies however do not protect completely against a challenge with the virus. Full protection is obtained only by concomitant
25 immunization with the F protein of PIV3.

Accordingly there still exists a need for antigens which will effectively protect against challenge with the PIV3 virus.

30 According to a first aspect of the present invention there is provided recombinant DNA encoding a fusion protein or an immunogenic derivative thereof comprising a portion of the F protein of PIV3 fused to a portion of the HN protein of PIV3.

35 The term immunogenic derivative as used herein encompasses any molecule which is a fusion protein which is immunologically reactive with antibodies raised to the fusion protein of the present invention or parts thereof or which elicits antibodies recognizing the fusion protein, the HN

- 3 -

- protein, the F protein or PIV3 virus. In particular immunogenic derivatives which are slightly longer or shorter than the fusion protein of the present invention may be used. Such derivatives may, for example, be prepared by substitution, addition, or rearrangement of amino acids or by chemical modifications thereof including those for enabling coupling of the fusion protein to other carrier proteins such as tetanus toxoid or Hepatitis B surface antigen. All such substitutions and modifications are generally well known to those skilled in the art of peptide chemistry.
- 10 Immunogenic fragments of the fusion protein which may be useful in the preparation of vaccines may be prepared by expression of the appropriate gene fragments or by peptide synthesis, for example using the Merrifield synthesis (The Peptides, Vol 2., Academic Press, New York, p3).
- 15 The recombinant DNA of the invention may form part of a vector, for example a plasmid, especially an expression plasmid from which the fusion protein may be expressed. Such vectors also form part of the invention, as do host cells into which the vectors have been introduced.
- 20 The invention further provides a fusion (or 'hybrid') protein or an immunogenic derivative thereof comprising a portion of the F protein of PIV3 fused to a portion of the HN protein of PIV3. Preferably the fusion is between an amino acid in the C-terminal part of a portion of the F protein and an amino acid at the N-terminus of a portion of the HN protein.
- 25 Preferably both the F protein and the HN protein components of the fusion protein of the invention lack a membrane anchor domain.
- 30 In one specific embodiment there is provided a fusion protein comprising amino acid residues 1 to 484 of the F protein fused to residues 88 to 572 of the HN protein (herein designated $Fs^{+a} \times HNa^{-}$).
- 35 In order to construct the DNA encoding a fusion protein according to the invention, cDNA containing the complete coding sequences of the F and HN proteins may be manipulated using standard techniques [see for example Maniatis T. et al Molecular Cloning, Cold Spring Harbor Laboratory, Cold Spring Harbor N.Y. (1982)] as further described hereinbelow.

- 4 -

In the course of carrying out such techniques recombinant DNA encoding fragments of the F and HN proteins may be obtained which further forms part of the present invention.

- 5 In particular DNA segments encoding the truncated protein Fs^+a^- (F protein lacking the membrane anchor domain) and the truncated protein HNa^- (HN protein lacking the 5' membrane anchor domain) are important intermediates.
- 10 A further construct which may be obtained is DNA encoding the HNa^- protein fused to a signal sequence from another gene, for example human myeloperoxidase (MPO). A particular embodiment is $SS_{MPO} \Delta HN$ corresponding to a hybrid containing the 22 amino acid signal peptide of MPO fused to the last 519 amino acid residues of the HN protein (aa 53 to
- 15 572).

Vectors comprising such DNA, hosts transformed thereby and the truncated or hybrid proteins themselves, expressed as described hereinbelow all form part of the invention.

- 20 For expression of the proteins of the invention, plasmids may be constructed which are suitable either for transfer into vaccinia virus or transfection into Chinese Hamster Ovary (CHO) cells or Vero cells. Suitable expression vectors are described hereinbelow.
- 25 For expression in vaccinia a vaccinia transfer plasmid such as pULB 5213 which is a derivative of pSC11 (Chakrabati *et al.* Molecular and Cellular Biology 5, 3403 - 3409, 1985) may be used. In one aspect the protein may be expressed under the control of the vaccinia P7.5 promoter.
- 30 For expression in CHO cells a glutamine synthetase (GS) vector such as pEE14 may suitably be used so that the protein is expressed under the control of the major immediate early promoter of human cytomegalovirus (hCMV-MIE). Alternatively a vector which allows the expression of the
- 35 coding module as a polycistronic transcript with the neo selection gene may suitably be used. In one preferred aspect the coding module is under the control of the Rous Sarcoma Long Terminal Repeat (LTR) promoter.

- 5 -

Preferably the plasmid for expression in CHO cells carries a DHFR expression cassette suitable for gene amplification using methotrexate.

5 Expression of full length HN in eukaryotic cells particularly CHO cells, is novel and forms a further aspect of the invention.

In order to define the invention more clearly reference is made to the appended drawings, in which:

10 Figure 1A shows a schematic representation of the Fs^+a^- cDNA module which was used to construct a plasmid pNIV3305;

Figure 1B shows a representation of plasmid pNIV3324 obtained by introducing the Fs^+a^- module into pULB 5213;

15 Figure 2A shows a representation of plasmid pNIV3308 carrying the sequence of HNa^- ;

Figure 2B is a representation of plasmid pNIV3325 obtained by
20 introducing the HNa^- module into pULB5213;

Figure 3 is a representation of plasmid pNIV3327 obtained by introducing the SS_{MPO} module into pULB5213;

25 Figure 4A is a representation of plasmid pNIV3310 obtained by introducing the $FxHN$ DNA sequence into pUC9;

Figure 4B is a representation of plasmid pNIV3326 obtained by
30 introducing the $FxHN$ DNA sequence into pULB5212;

Figure 5 is a representation of the Fs^+a^- coding module as subcloned in a pTDN vector to give plasmid pNIV3306 together with a representation of pRIT13572 containing the Fs^+a^- coding module subcloned into pEE14;

35 Figure 6 is a representation of the $Fs^+a^- \times HNa^-$ coding module recovered from pNIV3310 together with pRIT13571 containing the $Fs^+a^- \times HNa^-$ sequence subcloned into pEE14;

- 6 -

Figure 7 is a representation of the complete HN protein coding sequence from plasmid pIBI-HN together with pNIV3328 containing the HN coding sequence subcloned into pTNDPC2;

- 5 Figure 8 is a representation of the HNa⁻ coding module recovered from pNIV3308 together with pNIV3331 containing the HNa⁻ coding sequence subcloned into pTNDPC2;

- 10 Figure 9A is a representation of the pNIV3319 containing the HNa⁻ coding sequence subcloned into a pTDN vector;

Figure 9B shows the construction of plasmid pNIV3321 containing the SS_{MPO} Δ HN coding sequence from pNIV3319 and pNIV2706; and

- 15 Figure 9C shows the SS_{MPO} Δ HN sequence from pNIV3321 introduced into pTNDPC2 to form pNIV3330.

- In yet another aspect of the invention there is provided a vaccine composition comprising a protein according to the invention in
20 combination with a pharmaceutically acceptable carrier, a protein according to the invention for use in vaccinating a host and the use of a protein according to the invention in the preparation of a vaccine.

- Optionally, and advantageously, the PIV3 vaccine of the present
25 invention is combined with other immunogens to afford a polyvalent vaccine.

- In a particular aspect the invention further provides a vaccine composition comprising a protein according to the invention and an antigen against
30 respiratory syncytial virus (RSV) together with a suitable carrier or adjuvant. Antigens which stimulate production of antibodies against RSV in humans and animals are known in the art and examples are the major glycoprotein (G), fusion protein (F), phosphoprotein (P), nucleoprotein (N), and matrix (22K) protein of RSV A2 strain (a general review is given by
35 Stott, E.J. and Taylor, G. (1985) in Archives of Virology, 84, 1-52).

Vaccine preparation is generally described in New Trends and Developments in Vaccines, edited by Voller *et al*, University Park Press.

- 7 -

Baltimore, Maryland, U.S.A., 1978. Encapsulation within liposomes is described, for example by Fullerton, U.S. Patent 4,235,877.

In the vaccine of the present invention, an aqueous solution of the protein(s) can be used directly. Alternatively, the protein, with or without prior lyophilisation, can be mixed, absorbed or adsorbed with any of the various known adjuvants. Such adjuvants include, but are not limited to, aluminium hydroxide, muramyl dipeptide and saponins such as Quil A. Particularly preferred adjuvants are MPL (monophosphoryl lipid A) and 3D-MPL (3 deacylated monophosphoryl lipid A) [US patent 4,912,094]. A further preferred adjuvant is known as QS21 which can be obtained by the method disclosed in US patent 5,057,540. Use of 3D-MPL is described by Ribi *et al.* in *Microbiology* (1986) Levie *et al.* (eds) Amer. Soc. Microbiol. Wash. D.C., 9-13. Use of Quil A is disclosed by Dalsgaard *et al.*, (1977), *Acta Vet Scand.* 18, 349.

As a further exemplary alternative, the proteins can be encapsulated within microparticles such as liposomes or associated with oil-in-water emulsions. Encapsulation within liposomes is described by Fullerton in US patent 4,235,877. In yet another exemplary alternative, the proteins can be conjugated to an immunostimulating macromolecule, such as killed *Bordetella* or a tetanus toxoid. Conjugation of proteins to macromolecules is disclosed, for example by Likhite in patent 4,372,945 and Armor *et al.* in US patent 4,474,757.

The amount of the protein of the present invention present in each vaccine dose is selected as an amount which induces an immunoprotective response without significant, adverse side effects in typical vaccines. Such amount will vary depending upon which specific immunogen is employed and whether or not the vaccine is adjuvanted. Generally, it is expected that each dose will comprise 1-1000 µg of protein, preferably 1-200 µg. An optimal amount for a particular vaccine can be ascertained by standard studies involving observation of antibody titres and other responses in subjects.

The invention will now be illustrated by reference to the following examples.

- 8 -

Example 1 Vector constructionA) For transfer into vaccinia virus5 1. Fusion protein Fs⁺a⁻, lacking the membrane anchor region

Starting from plasmid pIBIcod, a cDNA clone encoding the complete F protein (received from Dr. K. Kimock, University of Ottawa, Canada), we reconstructed a cDNA module coding for the F protein lacking the
10 C-terminal membrane anchor sequence (Fs⁺a⁻).

Plasmid pIBIcod was digested by NsiI and BamHI to recover a 4016 bp DNA fragment corresponding to the vector and amino acid residues 1 to 377 of the F protein. On the other hand, a 362 bp NsiI-BamHI DNA piece
15 was generated by the polymerase chain reaction using plasmid pIBIcod as template; it corresponds to amino acid residues 378 to 493 of the F protein and includes a stop codon.

This fragment was ligated to the 4016 bp DNA piece obtained before to reconstruct a plasmid, pNIV3305, carrying the F protein lacking the
20 anchor domain (Fig. 1A).

The Fs⁺a⁻ module (1492 bp) was then recovered from pNIV3305 by digestion with HindIII-BamHI and introduced blunt-ended, in the correct
25 orientation for expression, into the SmaI site of the vaccinia transfer plasmid pULB5213, which is a derivative of the standard vaccinia vector pSC11 (Chakrabati *et al*, Molecular and Cellular Biology 5, 3403-3409, 1985). The resulting plasmid, pNIV3324, is illustrated in Fig. 1B.

30 2. Attachment protein HNa, lacking the 5' membrane anchor domain

Starting from plasmid pBI-HN, a cDNA clone containing the complete coding sequence of protein HN as well as its 3' non coding sequence (received from Dr.K. Kimock, University of Ottawa, Canada), we
35 reconstructed two different cDNA modules coding for the HN protein lacking the 5' membrane anchor domain. The first one, HNa consists of a strict deletion of amino acid residues 32 to 51 and the second one, SSMP_{PO} HN, consists of a fusion between a synthetic signal sequence derived from

- 9 -

the human myeloperoxidase cDNA (Moguilevsky *et al*, Eur. J. Biochemistry, 197: 605-614, 1991) and amino acid residues 54 to 572 of the HN protein.

- 5 a) The module coding for HNa⁺ was constructed as follows: plasmid pIBI-HN was digested with Asp718 and EcoRI to recover a 3935 bp DNA fragment corresponding to the vector and to amino acid residues 236 to 572 of the HN protein. In addition, two DNA fragments were generated by the polymerase chain reaction procedure using plasmid pIBI-HN as
10 template; the first one is 117 bp EcoRI-HindIII piece spanning the initiation ATG (Met1), the sequence coding for amino acids 2 to 31 and the codon for amino acid residue 52 of the HN protein. The second fragment is a 556 bp HindIII-Asp718 DNA piece encoding amino acid residues 53 to 235 of the HN protein.

15

- The three fragments obtained above were ligated to reconstruct a plasmid, pNIV3308 (Fig.2A) which carries the sequence of the HN protein lacking exclusively the anchor domain (sequence for amino acids 32 to 51). The HNa module (1688 bp) was then recovered from pNIV3308 by digestion
20 with XbaI and introduced blunt-ended into the SmaI site of the vaccinia transfer vector pULB5213 (see *supra*). The resulting plasmid, pNIV3325, is illustrated in Fig.2B.

- b) The module coding for SS_{MPO} Δ HN was constructed as follows:
25 starting from plasmid pNIV3325 (Fig 9B), which carries an hybrid sequence corresponding to the signal peptide (22 amino acids) of human myeloperoxidase fused to the last 519 amino acid residues of the HN protein (aa53 to 572), we excised a 1649 bp Bsp1286I-BamHI DNA fragment which, after blunting, was ligated into the SmaI site of the
30 vaccinia transfer plasmid pULB5213.

The resulting plasmid, pNIV3327, is illustrated in Fig. 3.

3. An hybrid Fs⁺a⁻xHNa⁺ protein

35

In this construction, the DNA coding for the HNa⁺ protein is fused downstream to the DNA encoding the Fs⁺a⁻ protein.

- 10 -

Plasmid pIBIcod (see supra) was digested with BamHI and Bg1II to recover a 4284 bp fragment corresponding to the vector and to amino acid residues 1 to 466 of the F protein. On the other hand, plasmid pIBI-HN (see supra) was digested with BamHI and AseI to recover a 1467 bp DNA
5 piece corresponding to amino acid residues 88 to 572 of the HN protein. At last, synthetic oligonucleotides (56/54 mers) were produced in order to provide the junction between the two fragments obtained above. These oligonucleotides are flanked by a Bg1II site on the 5' end and an AseI site on the 3' end, and code for amino acid residues 467 to 484 of the F protein.

10

The three DNA fragments were ligated to generate the plasmid pNIV3309. To facilitate further manipulation of the FxHN coding module, it was introduced into another vector, pUC9 (Pharmacia) to yield pNIV3310 (Fig.4A). The hybrid FxHN DNA sequence (2949bp) was
15 recovered from pNIV3310 by digested with BamHI and introduced by ligation into the Bg1II site of the vaccinia transfer plasmid pULB5212 (pULB5212 is identical to pULB5213 except for the orientation of the multisite linker sequence; see supra) to generate the final plasmid pNIV3326 (Fig.4B). This plasmid thus contains, under the control of the
20 vaccinia P7.5 promoter, an hybrid DNA sequence coding for 969 amino acids (residues 1 to 484 of the F protein fused to residues 88 to 572 of the HN protein.

B) For transfection into CHO cells

25

1. Fusion protein Fs⁺a⁻ lacking the membrane anchor domain

The module coding for Fs⁺a⁻, recovered from pNIV3305, was subcloned into the pTDN vector (Connors et al, DNA 7:651-660, 1988) in order to
30 provide appropriate flanking restriction sites for future constructions. The resulting intermediate plasmid, pNIV3306, was then digested with HindIII and SnaBI to recover the 1492 bp coding module. The DNA piece was ligated into the HindIII and SmaI sites of the glutamine synthetase (GS) vector, pEE14 (Cockett et al, Bio/Technology 8:662-667, 1990). The
35 resulting final plasmid, pRTT13572, contains the Fs⁺a⁻ protein under the control of the major immediate early promoter of the human cytomegalovirus (hCMV-MIE). The recombinant plasmid is illustrated in Figure 5.

2. The hybrid protein Fs^+a^-xHNa

The starting material pNIV3310, was digested with BamHI to recover the
5 module encoding the Fs^+a^-xHNa hybrid protein. This fragment was
inserted, blunt-ended and in the correct orientation for expression, by
ligation into the SmaI site of the pEE14 vector (see supra). The resulting
plasmid, pRIT13571, contains under the control of the hCMC promoter,
the sequence coding for the fusion between Fs^+a^- (aa residues 1 to 484)
10 and HNa^- (aa residues 88 to 572); it is illustrated in Fig. 6.

3. The attachment protein HN

Three constructions for expression in CHO cells have been made. The
15 first one, HN, corresponds to the full coding sequence of the attachment
protein, including the 5' membrane anchor domain. The second
construction is the HNa^- derivative, i.e. the attachment protein lacking
exclusively the anchor sequence (amino acid residues 31 to 52) and the
third construction, $SS_{MPO} \Delta HN$, is a fusion between the signal sequence
20 of human myeloperoxidase and a truncated form of the attachment
protein.

a) The full coding sequence for the attachment protein HN

25 The starting material, pIBI-HN (see supra), was digested with BamHI to
recover a 1736 bp DNA piece encoding the complete HN protein. The
fragment was ligated, blunt-ended, to the blunted HindIII and HpaI sites
of the eukaryotic expression vector pTNDPC2 (M.Reff, SmithKline
Beecham, Philadelphia, USA). This vector is a derivative of pTDN
30 (Connors et al, 1988, see supra) which allows the expression of a foreign
DNA as a polycistronic transcript with the neo selection gene. The
resulting plasmid, pNIV3328, thus carried the HN coding module
upstream to the neo gene under the control of the Rous Sarcoma Long
Terminal Repeat (LTR) promoter. In addition, the plasmid carried a
35 DHFR expression cassette suitable for amplification using methotrexate.
The plasmid is illustrated in Figure 7.

b) The HNa^- protein, lacking the membrane anchor domain

(residues 31 to 52)

The starting material, pNIV3308 (see *supra*), was digested with XbaI to recover a 1684 bp fragment encoding the HNa⁻ protein. The DNA piece
5 was ligated, blunt-ended, to the blunted HindIII and HpaI sites of the eukaryotic polycistronic expression vector pTNDPC2 (see *supra*). The resulting plasmid, pNIV3331, carries the HNa⁻ coding module upstream to the neo gene under the control of the RSVLTR promoter (Figure 8).

10 c) The SS_{MPO} Δ HN protein

The final plasmid for expression of SS_{MPO} Δ HN in CHO cells was obtained in three steps. The first one consists in introducing the
15 XbaI-XbaI 1684 bp coding cassette derived from pNIV3308 (see above, b) into the pTDN vector (Connors *et al*, 1988, see *supra*) to obtain the intermediate vector pNIV3310 (Fig.9A). From there, the actual construction of SS_{MPO} Δ HN was started. pNIV3319 was digested in SaI and EcoRI to recover a 7278 bp fragment corresponding to the nearly complete vector and to the HN amino acids 53 to 572. On the other hand,
20 plasmid pNIV2706 (Moguilevsky *et al*, Eur.J. Biochemistry 197:605-614, 1991) which encodes human myeloperoxidase, was digested with SaI and BglII to recover a 659 bp DNA piece carrying the RSV LTR promoter and the first 40 nucleotides coding for the signal peptide of human
25 oligonucleotide was generated to reconstruct the remaining sequence of the hMPO signal peptide. The three fragments obtained above were ligated together to yield the plasmid pNIV3321 (Fig. 9B).

The last step of the construction consisted of excising from pNIV3321 a
30 1649 bp Bsp1286I/BamHI DNA piece corresponding to the module coding for SS_{MPO} Δ HN and of introducing it, blunt-ended, into the blunted HindIII and HpaI sites of plasmid pTNDPC2 (see above). The final construct, pNIV3330, illustrated in Fig.9C, thus consists of a polycistronic eukaryotic expression vector carrying the SS_{MPO} Δ HN sequence fused
35 upstream to the neo gene and under the control of the Rous Sarcoma Virus Long Terminal Repeat promoter element.

Example 2

Expression in eukaryotic cellsA) via vaccinia virus recombinants

5

Recombinant transfer plasmids, pNIV3324, pNIV3325, pNIV3327 and pNIV3326, were transfected into vaccinia-infected CV-1 cells and recombinant viruses were isolated after Bromo-Uridine selection and plaque purification on the basis of their blue colour in the presence of X-gal. They will be referred to as VV3324, VV3325, VV3327 and VV3326 respectively. The human H143 fibroblast TK⁻ strain was used preferably to the RAT2 cells for plaque assays. The vaccinia virus used to infect cells was of the WR type (origin Borysiewicz, L.K.) for the VV3324, and the VR-119 type (origine ATCC) for VV3325, VV3327, and VV3326.

15

The procedure follows that one previously described for the obtention of vaccinia virus recombinants (Mackett, M. and Smith, G.L., J. Gen. Virology 67:2067-2082, 1986; Mackett, M., Smith, G.L. and Moss, B., J. Virology 49: 857-864, 1984).

20

1. Fusion protein Fs⁺a⁻, lacking the membrane anchor domain

The recombinant vaccinia virus, VV3324, was used to infect CV-1 cells in culture at a multiplicity of infection 1 (m.o.i.1). Infected cells (about 3.10⁵ per assay) and spent culture medium (about 2ml) were collected between 16 and 17 hours post infection. The presence of the Fs⁺a⁻ protein was identified by ELISA, Western blotting and immunoprecipitation on cell extracts and spent culture medium. For ELISA, a mixture of six monoclonal antibodies anti F (PIV3) was used to coat microtiter plates (Mab 4.454 - 4.478 - 4.549 - 4.519 - 4.726 - 4.789; E.Norrby, Stockholm) and rabbit anti PIV3 antiserum (E.Norrby, Stockholm) as capture antibody. Specific complexes were detected using alkaline phosphatase-conjugated affinity purified swine immunoglobulins to rabbit (Prosan) and an appropriate chromogenic substrate, according to standard procedures.

35

For Western blotting experiments, Concanavalin A concentrated spent culture medium were resolved by electrophoresis on 12%

- 14 -

SDS-polyacrylamide gels. After transfer onto nitrocellulose filters, separated proteins were probed with the rabbit anti PIV3 antiserum (E.Norrby, Stockholm). Complexes were detected using a swine anti-rabbit IgG conjugated to alkaline phosphatase (Prosan) and the appropriate chromogenic substrate, according to standard procedures.

Immunoprecipitation experiments were performed as follows. In short, the recombinant product was labelled *in vivo* by growing the recombinant vaccinia virus in the presence of ^{35}S -methionine. Labelled samples, cells extracts and medium, were incubated successively with the six monoclonal antibodies mixture anti F (PIV3) and rabbit anti-mouse; specific complexes were then recovered by binding to sepharose bound protein A. These procedures are well known to specialists in the art. Immunoprecipitates were analyzed onto 12% SDS-polyacrylamide gels which were then autoradiographed after drying.

The ELISA indicated that the recombinant Fs^+a^- protein is expressed in CV-1 cells infected with VV3324. The product was distributed between cell extracts and spent culture medium (Fig.10A). Western blotting analysis showed that the recombinant Fs^+a^- protein in concentrated spent culture medium migrates as the 70 kDa precursor (F_0) and as its subunit of 56 kDa (F_1). This result was confirmed on cell extracts by the immunoprecipitation experiments.

2. The attachment protein HN_{a} , lacking the 5' membrane anchor domain

The recombinant vaccinia virus, VV3325, was used to infect CV-1 cells in culture. All subsequent steps pertaining to the assays were as described *supra*, with the exception that a mixture of thirteen monoclonal antibodies anti HN (E.Norrby, Stockholm) was used as selective probe. (Rydbeck *et al.*, J.Gen.Virol. 67:1531-1542, 1986).

3. The attachment protein $\text{SS}_{\text{MPO}} \Delta \text{HN}$

The recombinant vaccinia virus VV3327 was used to infect CV-1 cells in culture. All subsequent steps were as described *supra*. The presence of the $\text{SS}_{\text{MPO}} \Delta \text{HN}$ protein was identified by ELISA. As seen in Fig.10B,

- 15 -

the majority of the product is selected into the spent culture medium although about one fifth of the total amount remains intracellularly.

4. The hybrid $Fs^{+}a^{-}xHNa^{-}$ protein

5

The recombinant vaccinia virus, VV3326, was used to infect CV-1 cells. All subsequent steps were as described supra. The data of the ELISA assay show that the majority of the recombinant $Fs^{+}a^{-}xHNa^{-}$ protein is secreted into the spent culture medium.

10

B) Expression in CHO cells (stable transformants)

1. The fusion protein $Fs^{+}a^{-}$, lacking the membrane anchor domain

15 The plasmid pRIT13572 was transfected by calcium phosphate co-precipitation into CHO-K1 cells, using 20 μ g DNA per $1,25 \times 10^6$ cells. The CHO-K1 cells were grown in GMEM-S medium. The GS transfectants were selected by addition of 25 μ M methionine sulfoximine (MSX) two days after transfection in GMEM-S medium lacking glutamine
20 and containing 10% dialysed FCS. Fresh medium replacements were performed each 4-5 days and 10 to 14 days later, resistant colonies were picked and transferred into 96 wells plates. Each transformant was then transferred into 24 wells plates and subsequently to 80 cm² flasks. The GS transformants were assayed for the $Fs^{+}a^{-}$ protein 3-4 days after
25 confluency. The procedure follows that one described in Cockett, M.I., Bebbington, C.R. and Yarranton, G.T., Bio/Technology 8:662-667, 1990). Cell culture supernatants and extracts were assayed using ELISA and Western blotting as described supra.

30 Gene amplification procedures were done as described previously (Cockett et al., see supra). The distribution of the $Fs^{+}a^{-}$ protein between cell extract and supernatant of clones obtained during the first screening was measured. The data of the ELISA show that about 80%-90% of the product is secreted into the medium.

35

2. The hybrid protein $Fs^{+}a^{-}xHNa^{-}$

Procedures for expression of the hybrid $Fs^{+}a^{-}xHNa^{-}$ protein in CHO cells

- 16 -

transfected with plasmid pRIT13571 followed those described above. Cell culture supernatants and extracts were assayed using ELISA and Western blotting. Gene amplification procedures were done as described previously (Cockett *et al.*, see *supra*).

5

The distribution of the hybrid $Fs^{+}a^{-}xHN^{+}$ protein between cell extract and supernatant of a representative cell line $Fs^{+}a^{-}xHN^{+}$ clone 6 obtained by an ELISA assay was measured. The product was almost evenly distributed between cells and medium.

10

3. The attachment protein HN (complete protein)

Plasmid pNIV3328 was introduced by electroporation into CHO cells. Selection of recombinant cell lines was done using geneticin (G418) and amplification of cell lines was performed using methotrexate. All procedures used follow those described in Moguilevsky *et al.*, (Eur.J.Biochemistry, 197: 605-614, 1991).

15

G418^R clones were obtained and assayed for the production of the full size HN protein using the systems described before. Clones shown to produce the recombinant protein were amplified with methotrexate at different concentrations and retested for production. ELISA assays performed on the amplified cell lines showed that the recombinant HN protein was produced and that it accumulated into the CHO cells.

20

4. The attachment protein HN^{+} , lacking the membrane anchor domain

Plasmid pNIV3331 was introduced by electroporation into CHO cells. All subsequent steps were as described *supra*.

25

Random amplification of G418 resistant clones produced cell lines accumulating the recombinant protein, as identified by ELISA.

35 5. The attachment protein $SS_{MPO} \Delta HN$

Plasmid pNIV330 was introduced by electroporation into CHO cells. All subsequent steps were as described *supra*. G418^R clones were assayed by

- 17 -

ELISA for the production of the SS_{MPO} HN protein; some of the clones were positive in the assay and were amplified with various concentrations of methotrexate. Amplified cell lines were shown to secrete the recombinant protein into the culture medium. The ELISA assay shows that the distribution of the SS_{MPO} Δ HN recombinant product between cell extract and supernatant of a representative amplified cell line SS_{MPO} Δ HN (clone 3B) is about 90% in the medium and 10% in the cells.

10

15

20

25

30

35

40

45

- 18 -

CLAIMS

1. A fusion protein or an immunogenic derivative thereof comprising a portion of the F protein of PIV3 fused to a portion of the HN protein of
5 PIV3.
2. A fusion protein or an immunogenic derivative thereof as claimed in claim 1, wherein the fusion is between an amino acid in the C-terminal part of a portion of the F protein and an amino acid at the N-terminus of a
10 portion of the HN protein.
3. A fusion protein or an immunogenic derivative thereof as claimed in claim 1 or 2, wherein both the F protein and the HN protein components of the fusion protein of the invention lack a membrane anchor domain.
15
4. A fusion protein or an immunogenic derivative thereof as claimed in any preceding claim comprising amino acid residues 1 to 484 of the F protein fused to residues 88 to 572 of the HN protein.
- 20 5. Recombinant DNA encoding a fusion protein or an immunogenic derivative thereof as defined in any one of claims 1 to 4.
6. Recombinant DNA encoding a truncated protein or an immunogenic derivative thereof comprising the F protein lacking the anchor protein
25 domain or the HN protein lacking the 5' membrane anchor domain.
7. Recombinant DNA encoding the HNa⁺ protein or an immunogenic derivative thereof fused to a signal sequence from another gene.
30
8. Recombinant DNA as claimed in claim 7 wherein the signal sequence comprises a hybrid protein sequence containing the 22 amino acid signal peptide of MPO fused to the amino acid residues 53 to 572 of the HN protein.
35
9. An expression vector comprising recombinant DNA as defined in any one of claims 5 to 8.

- 19 -

10. A host transformed with a vector as defined in claim 9.
11. A vaccine composition comprising a protein as defined in any one of claims 1 to 4 or an immunogenic derivative thereof in admixture
5 with a pharmaceutically acceptable carrier.
12. A vaccine composition as claimed in claim 11 which further comprises an antigen effective in stimulating production of antibodies to respiratory syncytial virus.
- 10 13. A vaccine composition as claimed in claim 11 or claim 12 further comprising 3D Monophosphoryl lipid A or QS-21.
14. A vaccine composition as claimed in any one of claims 11 to 13
15 wherein the carrier is an oil-in-water emulsion.
15. A fusion protein or an immunogenic derivative thereof as defined in any one of claims 1 to 4 for use in medicine.
- 20 16. A vaccine composition as defined in any one of claims 11 to 14 for use in medicine.
17. A process for the production of a fusion protein as defined in any one of claims 1 to 4 which process comprises expressing a DNA sequence
25 encoding said protein in a host cell and recovering the protein.
18. A method of treating a human or animal susceptible to PIV3 infections comprising administering an effective amount of a vaccine as defined in any one of claims 11 to 14.
- 30 19. A process for the production of full length HN comprising expressing a DNA sequence encoding full length HN in a eukaryotic cell and recovering the protein.
- 35 20. A process as claimed in claim 19 wherein the eukaryotic cell is a CHO cell.

- 20 -

21. Use of a protein or an immunogenic derivative thereof as defined in any one of claims 1 to 4 in the manufacture of a medicament for use in the treatment of viral infections.

5

10

15

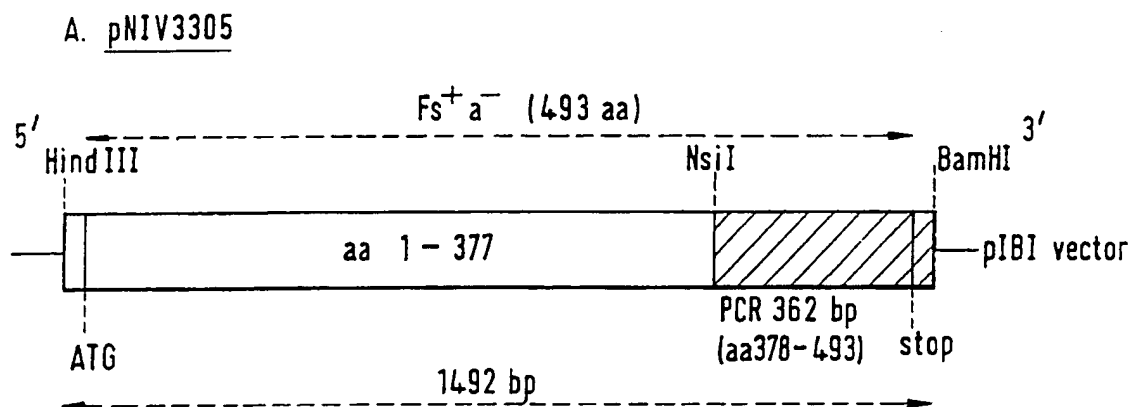
20

25

30

35

1/11



Primer 1 5' $\overset{378}{\text{GATATGCA}}\text{TTTGTCAATGGAGGAG}$ 3'

NsiI

Primer 2 3' GTAGTTAGATCGTGGTGTATCCTAGGG 5'

493 stop BamHI

Fig.1A

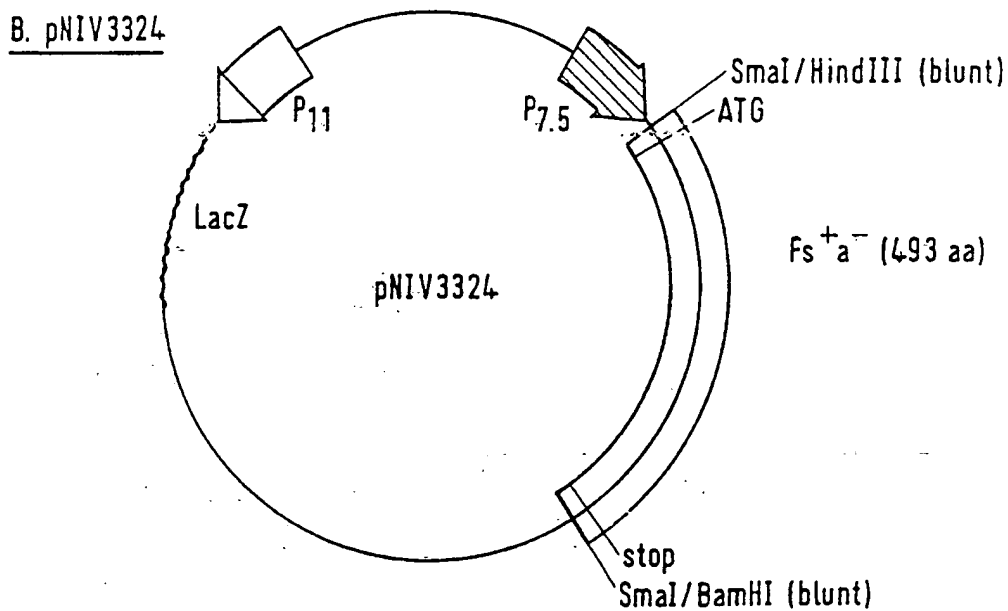
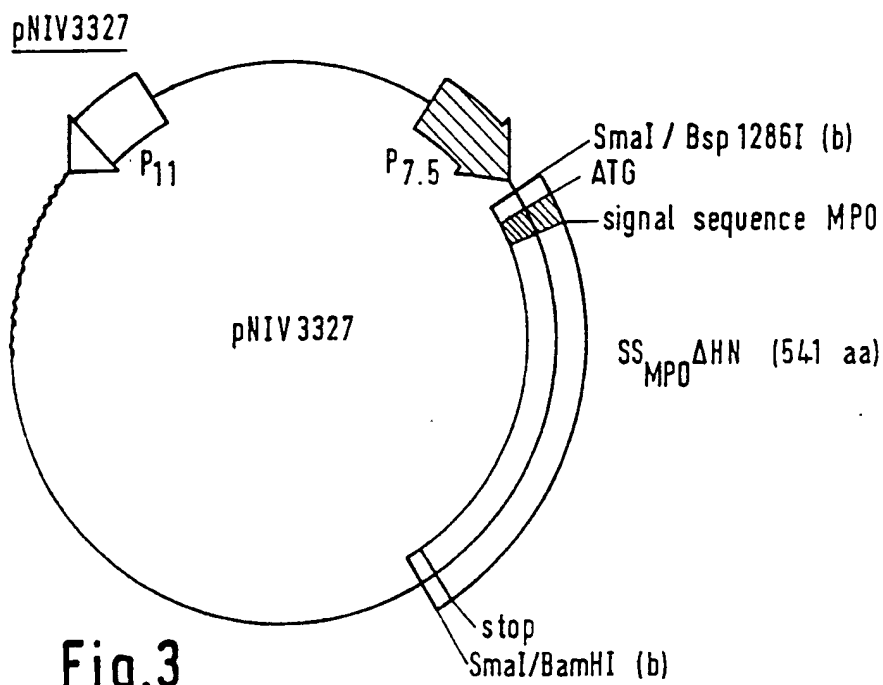
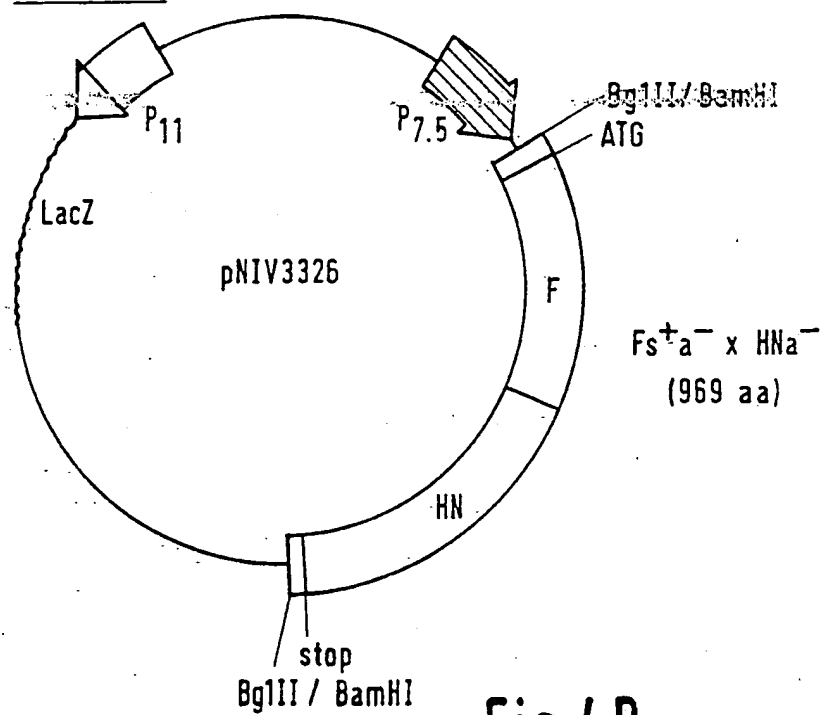


Fig.1B

3/11



B. pNIV3326



4/11

A. pNIV3310

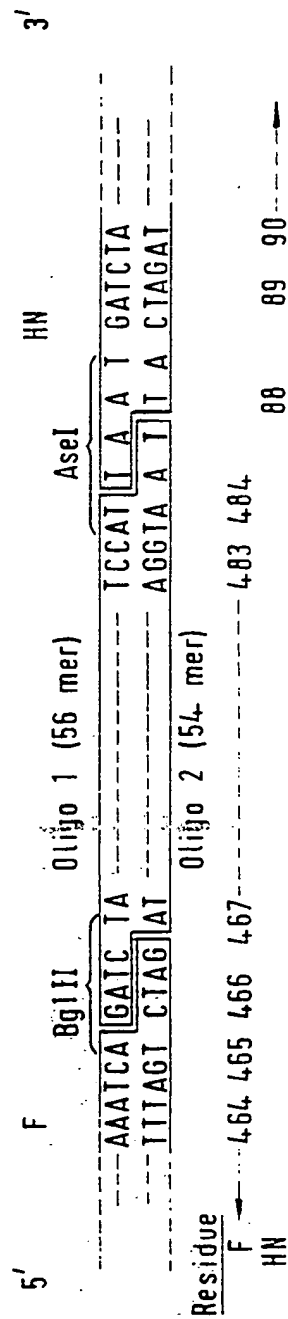
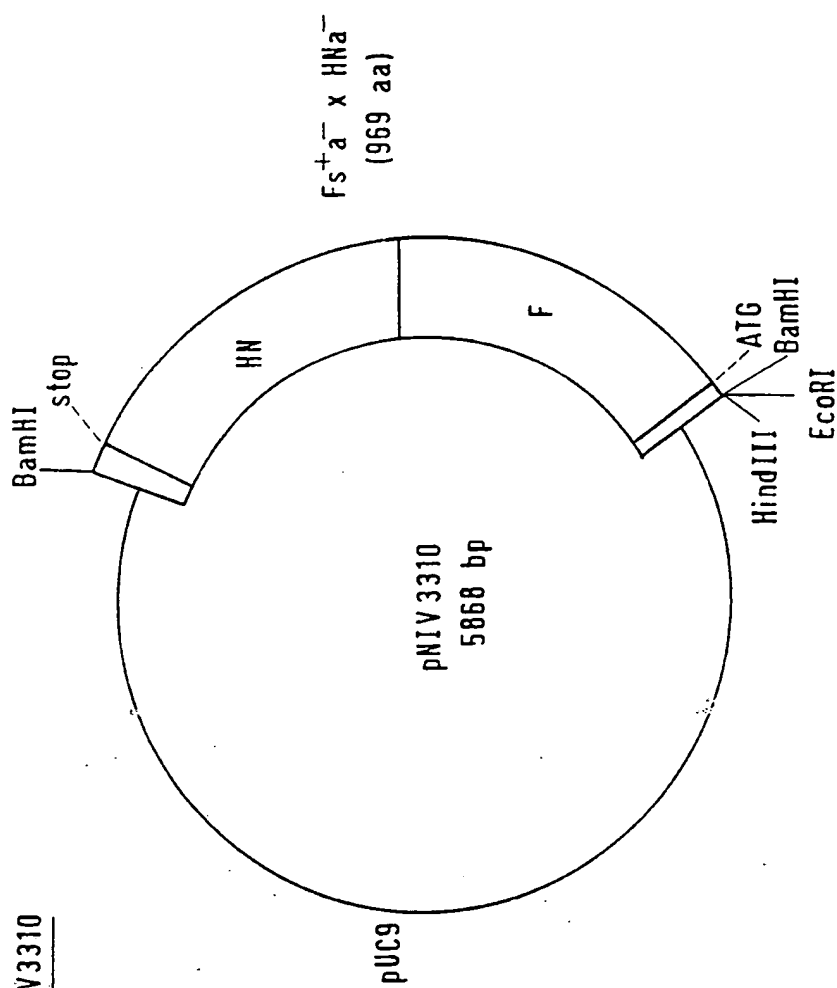


Fig.4A

5/11

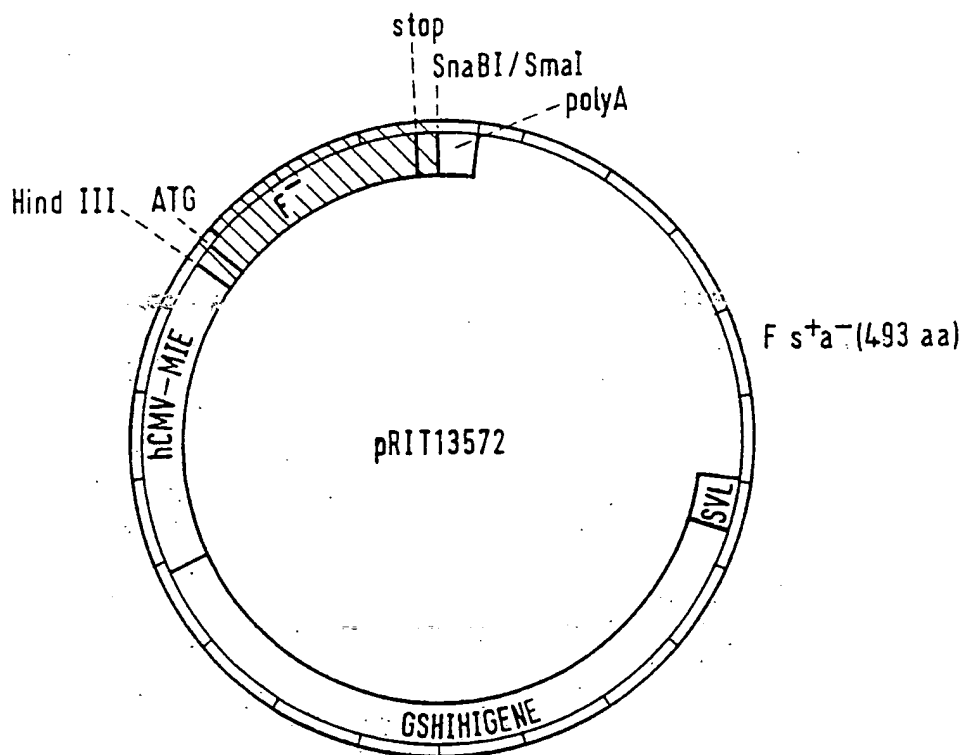
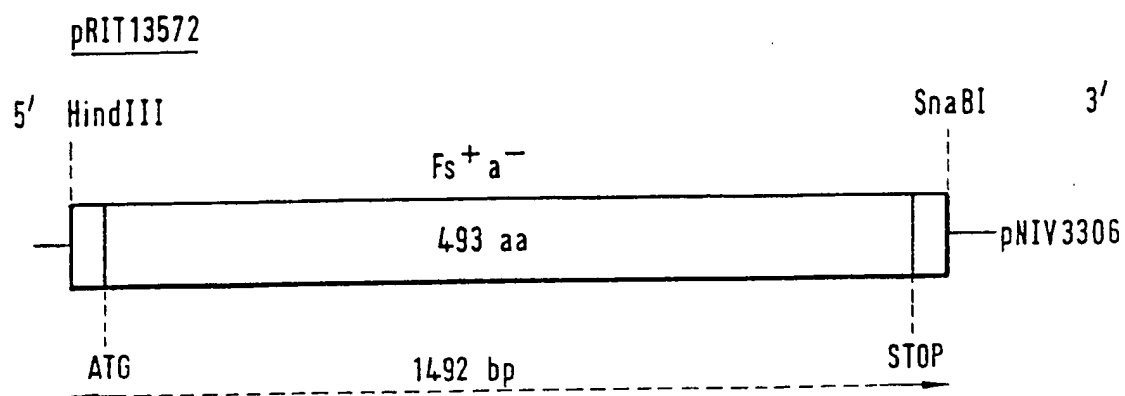


Fig.5

6/11

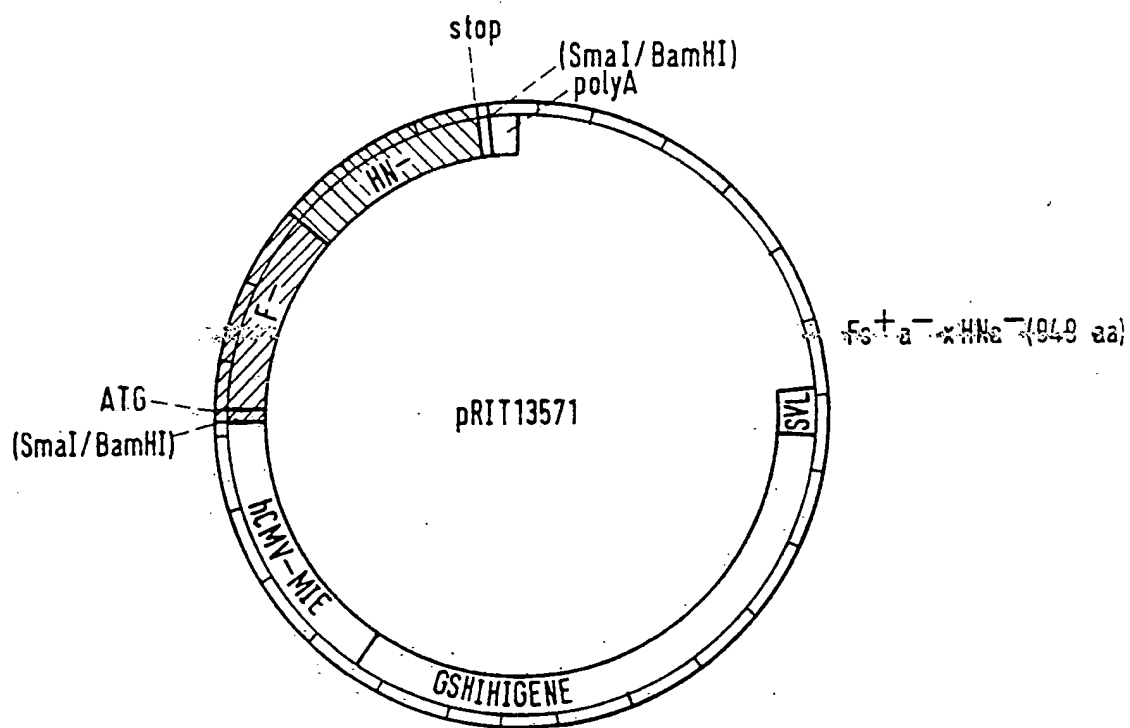
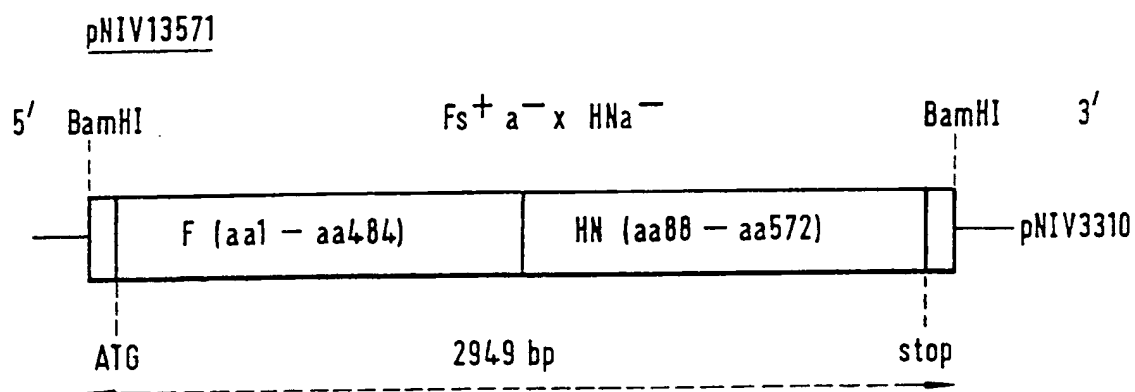


Fig.6

7/11

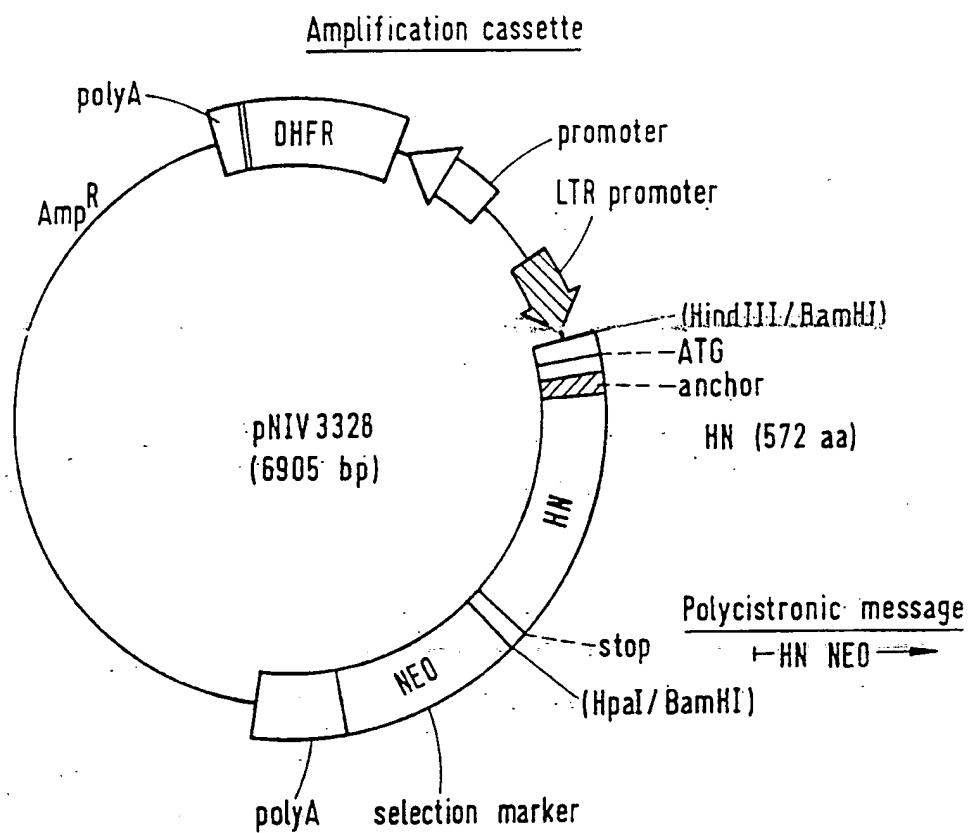
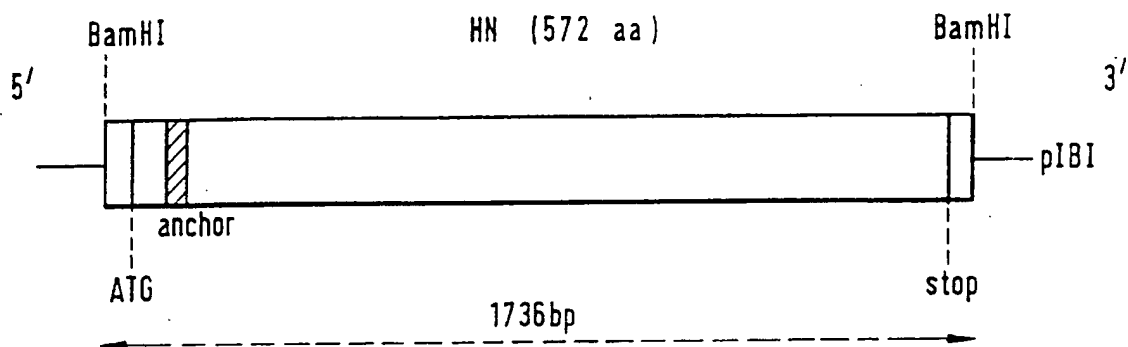


Fig.7

8/11

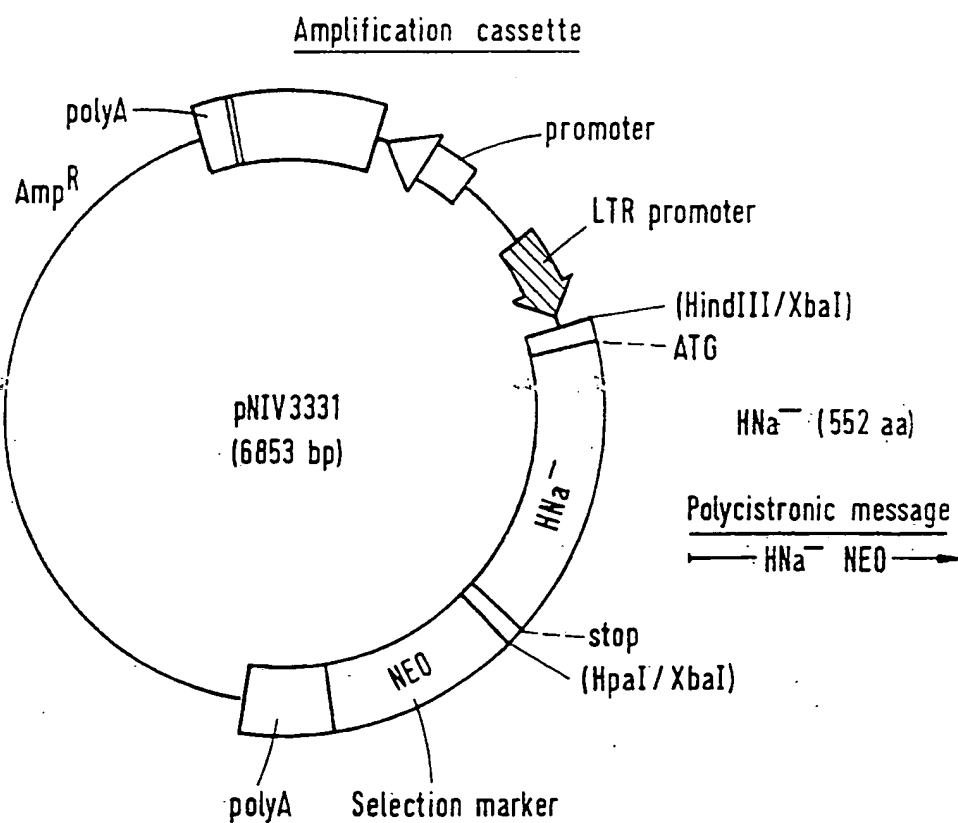
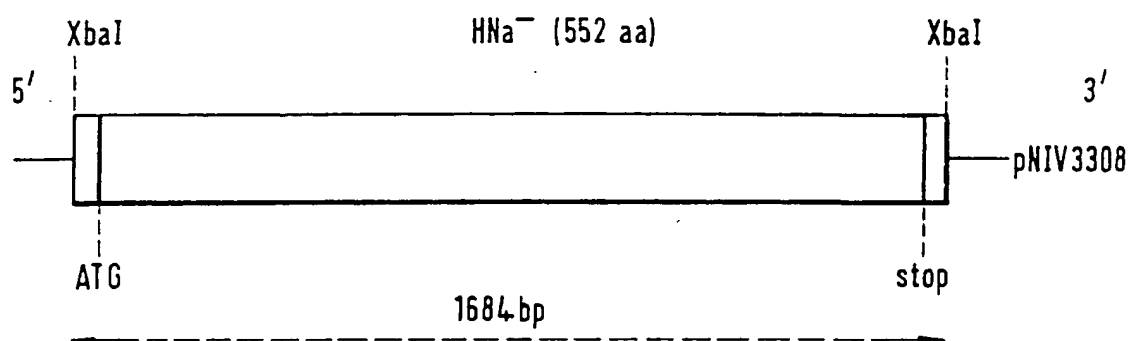


Fig.8

9/11

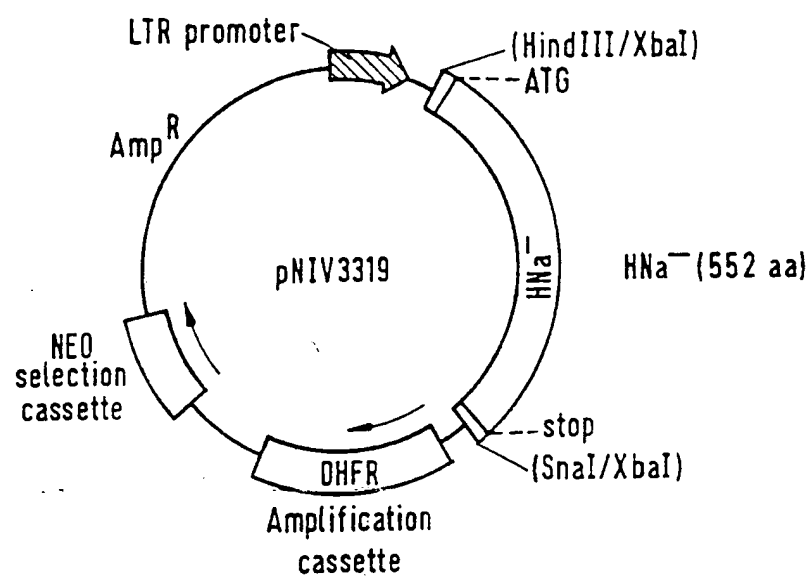
A. pNIV3319

Fig.9A

10/11

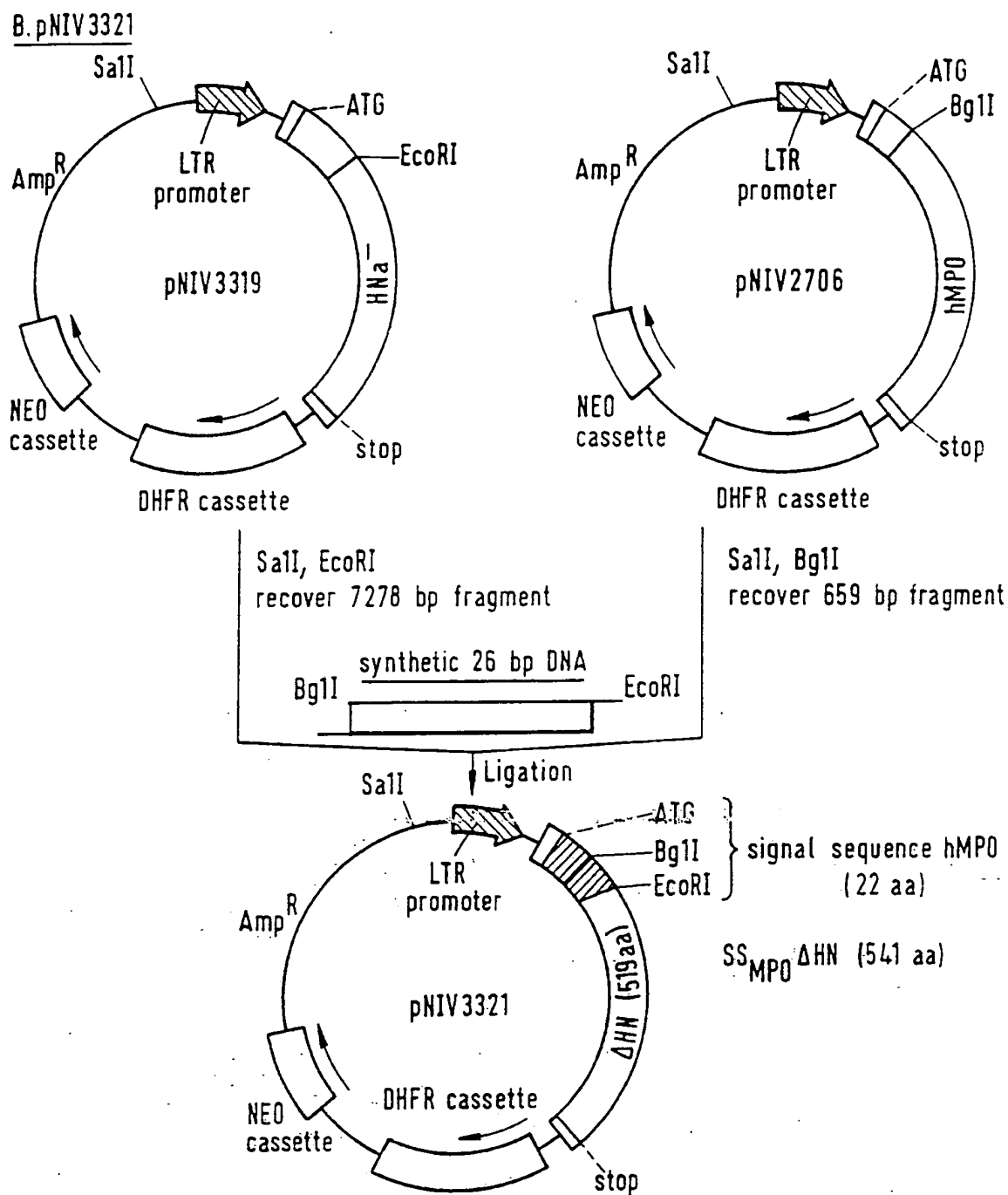


Fig.9B

11/11

C. pNIV3330

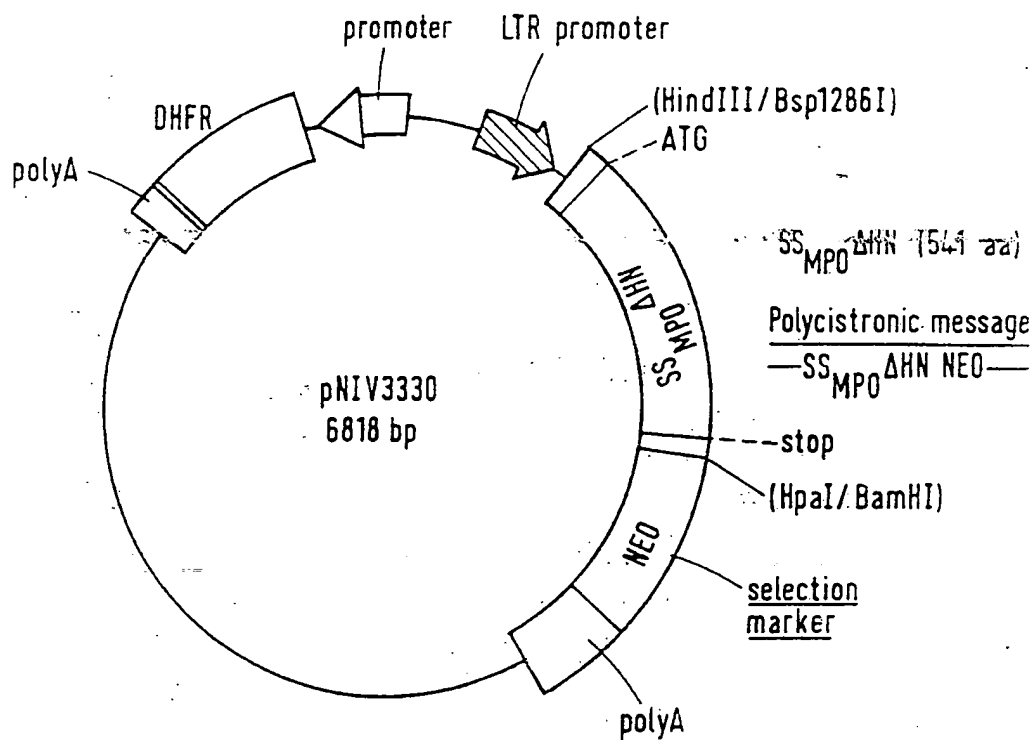
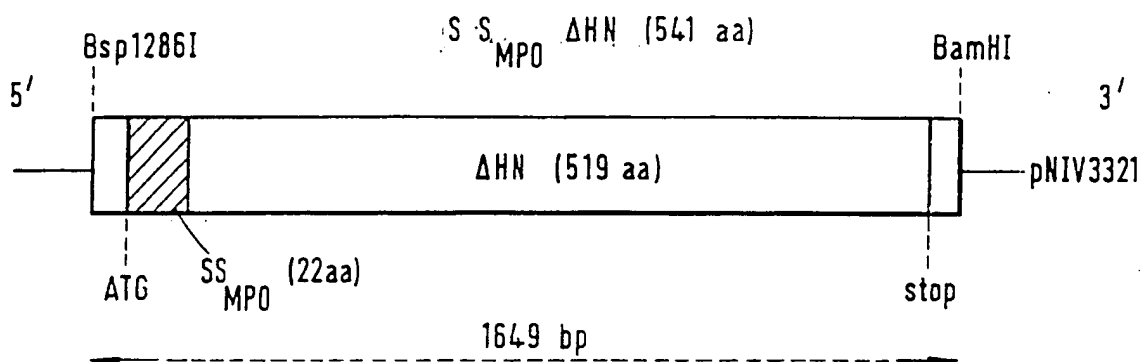


Fig.9C

THIS PAGE BLANK (USPTO)